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DETERMINATION OF TOCOPHEROLS AND TOCOTRIENOLS IN VEGETABLE OILS AND FATS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Results of a collaborative study and the standardised method

Prepared for publication by

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* Membership of the Commission during the period of the collaborative study (1983–87) was as follows:

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Determination of tocopherols and tocotrienols in vegetable oils and fats by high performance liquid chromatography: results of a collaborative study and the standardised method

Abstract - The development, by collaborative study, of a standardised method for the determination of tocopherols and tocotrienols in vegetable oils and fats is described. The procedure involves separation of the individual tocopherols and tocotrienols by the direct high-performance liquid chromatography (HPLC) of a solution of the oil or fat in an organic solvent. The application of the procedure to processed products such as margarines containing added tocopherol esters was also studied.

INTRODUCTION

Tocopherols are integral components of the unsaponifiable matter present in most vegetable oils and fats; tocotrienols occur at significant levels in palm oil. Both tocopherols and tocotrienols have important antioxidant and nutritional properties and alpha-tocopherol is being increasingly used as a food additive in prepared fats. The biological significance of the tocopherols (Vitamin E) in human nutrition is too well documented to warrant discussion here. At the time of the initiation of the study (1983) it was considered that the IUPAC published standard method (2.404) for tocopherols, which is based on thin-layer chromatographic isolation of the tocopherols followed by quantitative estimation using gas-liquid chromatography, was time consuming and somewhat restricted in scope as respects the separation of all of the commonly occurring tocopherols. Furthermore, the rapid advance in the design of HPLC instrumentation, together with the fact that analyses using HPLC techniques were being carried out on a regular basis in many laboratories, had emphasised the desirability of standardising a procedure based on HPLC.

1st (PRELIMINARY) COLLABORATIVE STUDY AND RESULTS

In the 1983/84 preliminary study a blended soya/maize oil (being developed as a reference material - see Acknowledgements) was analysed by thirteen laboratories. The protocol provided for the study allowed participants a measure of freedom as to the choice of chromatographic conditions to be used. Six sets of results obtained using fluorescence detection and eight sets using ultra violet detection were submitted. For reasons of space the results obtained are not reproduced in this report.

Fluorescence detection enabled the tocopherols of interest to be well resolved from other unidentified peaks, whilst the chromatograms obtained by UV detection generally contained more interfering peaks. The amount of sample and standard loaded on to the analytical columns highlights the fact that fluorescence detection is at least ten times more sensitive than UV detection.

This preliminary study was regarded as having been most useful. It demonstrated that fluorescence detection is to be preferred for the determination of tocopherols but that UV detection can also be used satisfactorily, although in this case the choice of mobile phase composition and column packing material is more critical. The various mobile phase compositions, flow rates, and column types used by participants in conjunction with fluorescence detection all produced satisfactory chromatograms but chromatograms obtained by UV detection contained peaks other than those due to tocopherols.

It was therefore decided to continue the study during the following year with a protocol having stricter control over the chromatographic conditions. In particular, it was recommended that specific requirements should be set out in the protocol regarding the propan-2-ol content of the mobile phase and the flow rate of the latter; it was also considered important to provide a sample of palm oil for the next study so that the performance of the method for the determination of tocotrienols could also be also assessed.

2nd COLLABORATIVE STUDY AND RESULTS

For the second study (1984/85) samples of soya and wheat germ oils were provided, as these contain significant levels of alpha-, gamma-, delta-tocopherols, and alpha-, beta-, delta-tocpherols, respectively. Samples of palm oil (one each of crude and refined) were also circulated since these oils contain mainly alpha-tocopherol together with significant

amounts of alpha- and gamma-tocotrienols. The participants were recommended to use a 250 mm x 4 mm HPLC column packed with microparticulate silica such as LiChrosorb SI 60 5 µm or 5 µm Spherisorb silica; if a fluorescence detector was available the excitation wavelength was to be set at 290 nm and the emission wavelength at 330 nm. Alternatively, UV detection at 292 nm was recommended. The mobile phase was to be hexane - propan-2-ol (99.5/0.5 v/v) with a flow rate of about 1 ml/min. The concentration (expressed as µg/ml) of a prepared standard alpha-tocopherol solution (10 mg in 100 ml of propan-2-ol) was to be determined by measuring its absorbance at 292 nm and dividing the observed value by a prescribed factor of 0.0076.

Results were received from seventeen laboratories and the statistical evaluation of these results, for alpha-tocopherol only, (carried out according to ISO 5725) are given in Table 1. For comparative purposes the results obtained by the two methods of detection (fluorescence and UV) have been tabulated separately.

Examination of the raw data submitted indicated that a few laboratories had erroneously identified some of the tocopherols, e.g. results for beta-tocopherol were in some cases reported as gamma-tocopherol and tocotrienols were reported by several laboratories as tocopherols. Appropriate corrections were made to the raw data before the statistical analyses were carried out.

TABLE 1. STATISTICAL ANALYSIS OF RESULTS FOR ALPHA-TOCOPHEROL (1984/85)

Sample	crude palm oil		refined palm oil	
Detection	Fl	UV	Fl	UV
Number of laboratories	6	4	5	4
Number of results	12	8	10	8
Number of accepted results	12	8	10	8
MEAN VALUE (µg/g)	161	140	109	87
Repeatability standard deviation (S_r)	6.29	13.14	3.98	6.88
Repeatability coefficient of variation	3.9%	9.3%	3.6%	7.9
REPEATABILITY VALUE (r) [2.83 x S_r]	18	37	11	19
Reproducibility standard deviation (S_R)	61.2	53.3	29.8	26.9
Reproducibility coefficient of variation	38.0%	38.9%	27.3%	30.8%
REPRODUCIBILITY VALUE (R) [2.83 x S_R]	173	151.1	84	76

Sample	soya oil		wheat germ oil	
Detection	Fl	UV	Fl	UV
Number of laboratories	5	3	5	4
Number of results	10	6	10	8
Number of accepted results	10	6	10	8
MEAN VALUE (µg/g)	87	80	152	81
Repeatability standard deviation (S_r)	7.5	5.5	2.9	7.6
Repeatability coefficient of variation	8.6%	6.8%	1.9%	9.3
REPEATABILITY VALUE (r) [2.83 x S_r]	21	15	8	21
Reproducibility standard deviation (S_R)	14.9	26.5	32.7	31.7
Reproducibility coefficient of variation	17.1%	33.1%	21.6%	39.0%
REPRODUCIBILITY VALUE (R) [2.83 x S_R]	42	75	93	90

Note:

Fl = fluorescence detection; UV = ultra violet detection

The repeatability for all results appeared to be satisfactory, but the reproducibility was poor. However, it should be borne in mind when considering reproducibility that laboratories were measuring labile compounds using different types of labile standards. Some excellent chromatograms were submitted with results in this second study, thereby demonstrating that the chromatography of the tocopherols and tocotrienols could be optimised. Fluorescence detection produced more sharply defined peaks, although this is not reflected in the statistical results. Although results for tocotrienols were encouraging, it was clear that some participants had difficulty with the identification of these components on the chromatograms. It was decided to continue the study during 1985/86 with a revised protocol which took into account the experience gained in the study and to pay particular attention to providing guidance for the identification of the tocopherols.

TABLE 2. RESULTS FOR TOCOPHEROLS expressed in µg/g (1985/86)

(means of results from duplicate injections)

Lab.	Sample 1				Sample 2				Sample 3				Sample 4			
No.	alpha	beta	gamma	delta	alpha	beta	gamma	delta	alpha	beta	gamma	delta	alpha	beta	gamma	delta
01	96	30	595	244	77	309	349	140	157	23	750	135	209	---	---	---
02	64	11	519	301	94	223	338	188	144	10	659	186	195	---	---	---
03	328*	15	501	227	1259*	243	302	95	83	7	591	116	212	---	205	35
04a	60	11	512	230	68	208	280	104	114	6	574	117	209	---	---	---
04b	70	16	515	268	79	252	236	123	129	10	681	138	189	---	---	---
05	76	12	496	238	57	205	225	102	132	10	567	128	205	---	---	---
06	71	17	498	245	59	219	268	118	133	12	589	126	197	---	---	---
07a	64	17	498	249	63	234	279	124	108	9	552	125	171	---	209	45
07b	71	19	504	-	56	214	236	-	101	11	519	-	168	---	170	-
08	47	-	722*	330	90	177	157	-	90	-	928*	201	91	---	220	44
09	66	64	501	230	194*	-	-	-	142	53**	564	121	191	---	195	43
10(S)	113	20	468	262	---	189	291	130	205	18	548	141	386*	---	181	42
11	51	16	446	331	6*	113	129	143	37	8	566	154	108	---	174	57
12	46	26	462	231	---	121	139	123	96	12	563	118	21*	---	201	32
13a	52	22	557	237	64	323	303	128	100	32	670	143	173	---	170	37
13b	64	21	564	245	64	335	313	131	104	30	666	144	150	---	169	36
14	48	12	318	175	--	184	162	136	112	8	401	42	197	---	98	11
15	50	15	473	371	3	178	232	171	100	12	600	210	194	---	272	91
16	243	18	458	150	61	234	613*	72	115	11	610	87	145	---	133	31
17a	89	13	523	265	15	211	271	120	156	10	595	137	265	---	224	34
17b	107	20	527	248	18	206	245	137	198	17	613	140	289	---	221	54
18	154	77	565	240	426	284	294	1631	152	62	711	256	165	---	22	60
19	69	13	531	279	87	221	283	120	118	9	652	153	212	---	198	35

Notes: *results rejected on technical grounds; Labs 04 & 07 results by two columns;

Lab 8 not included in statistical analysis; **duplicates Cochran outlier;

Lab 10 (single results not included in statistical analysis); 14 and 16 (some results* rejected on technical grounds), not included in statistical analysis; Lab 13 results in duplicate; Lab 17 results by two columns;

Labs 18, 19 received too late for inclusion in statistical analysis;

3rd COLLABORATIVE STUDY AND RESULTS

For the 1985/86 study four samples were distributed, their composition being designed to provide tocopherol and tocotrienols levels covering the range likely to be encountered in vegetable oils. Sample 1 was a refined soya oil; Sample 2 a 1:1 blend of wheat germ oil and soya oil; Sample 3 a 1:1 blend of soya oil and maize oil; Sample 4 was a refined palm oil specifically included for tocotrienols.

In addition, two reference samples of blended oils were provided to assist in the identification of the tocopherols: 1) a 2:1:2 blend of soya/maize/ wheat germ oil and 2) a refined palm oil identical to Sample 4; each participant was provided with copies of chromatograms (obtained in the co-ordinator's laboratory) indicating the HPLC separation of the different tocopherols and tocotrienols present in the reference samples.

Although some participants in the 1984/5 study had suggested that reverse-phase columns could be adopted for the chromatography, this was not pursued since it had been demonstrated that beta- and gamma-tocopherols could not be separated when using reversed-phase materials. However experience with Partisil PAC (which consists of amino and cyano groups) had been reported [ref 1] as acting as a highly polar stationary phase when used with hydrophobic mobile phases and participants in the 1985/86 study who had access to a Partisil PAC column were accordingly invited to submit results using this column, although this was not to be considered as constituting a part of the collaborative study. Fig 1 shows the quality of separation of the tocopherols which can be obtained when using this type of column.

Results received from nineteen laboratories during this 3rd study are summarised in Table 2. Table 3 gives a statistical analysis of seventeen sets of results and in Table 4 details will be found of the HPLC operating parameters adopted by the participating laboratories. Although the coefficient of variation values for repeatability could be considered to be reasonably satisfactory, it will be noted that those for the reproducibility tend to be higher than the 16% value that has been suggested as being the maximum acceptable for the determination of analytes at the ppm (µg/g) level [ref 2]. Nevertheless the results indicated that a significant number of laboratories could obtain very satisfactory results using the method studied and it appeared that little would be achieved by repeating the study.

One of the recurring problems encountered by the participants appeared to be the availability of alpha-, beta-, gamma- and delta-tocopherol standards. Several participants contacted the co-ordinator regarding problems of supply, and only half of the participants were able to use all four standards. Some used one of the reference oil samples provided to calibrate their procedure after the co-ordinator had supplied details of the tocopherol content as determined in his laboratory. One laboratory reported that they had prepared standard tocopherols by TLC clean-up of a mixture of tocopherols intended for industrial use. Several laboratories who used UV detection without access to all of the standards, normalised the response of the tocopherols to the alpha form for quantitation purposes. One participant drew attention to a literature report stating that the purity of Merck tocopherol standards may fall between 85% and 100% [ref 3]. This confirmed the importance of determining the concentration of prepared tocopherol standard solutions by UV spectrometry.

An anomaly in the protocol arose from the fact that instructions were given for the samples to be dissolved in hexane prior to chromatography. It was requested however that tocopherol standards should be dissolved in propan-2-ol so that their concentrations could be determined by UV spectrometry. This solution was to be subsequently diluted with hexane prior to chromatography, but the relatively large amount of alcohol present gave rise to peak broadening and non-reproducible retention times.

The requirement for dissolving the standards in propan-2-ol originated from the fact that at the time UV absorbance data for the tocopherols was only available with respect to their solutions in methanol and in propan-2-ol. In the co-ordinator's laboratory subsequent measurements of the absorbance of the four tocopherol standards at 292 nm in methanol and in hexane were made and the absorbances were found to be significantly higher when measured in hexane as opposed to methanol; furthermore the absorbance maxima in hexane shifted to higher wavelengths and the shape of the absorption profile was somewhat distorted. These facts make it undesirable to determine the concentration of tocopherol standards in hexane. In view of this it was decided to modify the experimental protocol so that standards would be required to be initially prepared in hexane. An aliquot will then be taken, the hexane removed in the presence of nitrogen in low actinic glassware, alcohol added and the concentration subsequently determined by UV spectrometry.

Chromatograms obtained using UV detection were again found to be generally more complex than those obtained when fluorescence detection is used, again stressing the need for special care in the selection of column working parameters when using UV detection. Several participants suggested the use of 3-tert-butyl-4-hydroxyanisole (BHA) as an internal standard but in the co-ordinator's view the correct use of an HPLC loop-injector using external calibration, should provide equally reliable results. After studying some of the chromatograms submitted, especially from participants who used UV detection, it was evident that components of some oils may co-elute with an internal standard such as BHA and thus give rise to erroneous results.

TABLE 3. STATISTICAL ANALYSIS OF RESULTS FOR TOCOPHEROLS and TOCOTRIENOLS (1985/86)

Sample	1	1	1	1	2	2	2	2
Tocopherol	alpha	beta	gamma	delta	alpha	beta	gamma	delta
No of labs	17	16	17	16	15	16	16	14
No accepted	30	28	32	26	18	30	30	28
MEAN VALUE (µg/g)	69	17	508	263	69	217	225	130
S_r	3.5	0.8	12.6	5.3	4.3	5.8	13.0	6.3
CV_r	5%	5%	2.5%	2%	6%	3%	5%	5%
(r)	10	2	36	15	12	16	37	18
S_R	17.5	5.1	36.0	44.5	12.8	56.1	62.4	25.8
CV_R	25%	31%	7%	17%	19%	26%	24%	20%
(R)	49	15	102	126	36	159	177	73
Sample	3	3	3	3	4*	4*	4*	4*
Tocopherol	alpha	beta	gamma	delta	alpha	beta	gamma	delta
No of labs	17	17	17	16	16	-	16	15
No accepted	34	32	32	28	32	-	30	30
MEAN VALUE (µg/g)	124	12	603	134	203	-	213	45
S_r	7.1	0.9	18.8	4.3	13.2	-	12.0	3.7
CV_r	6%	7%	3%	3%	7%	-	6%	8%
(r)	20	2.5	53	12	38	-	34	8
S_R	29.9	6.7	60.4	53.8	45.8	-	30.3	17.7
CV_R	24%	56%	10%	40%	23%	-	14%	39%
(R)	84	19	171	152	130	-	86	39

*results for tocotrienols

TABLE 4. HPLC OPERATING PARAMETERS (1985/86)

Lab. No.	Mobile Phase Hexane/Propanol (99.5:0.5 v/v)	Flow rate ml/min	Column Dimensions length mm	int. dia. mm	Column (standard Lichrosorb SI 60 5 µm)	Detection UV nm	Fl Ex	Fl Em
01	standard	1.0	250	4.6	not stated	-	295	330
02	standard	1	250	4.6	Nucleosil 100 5 µm	-	290	330
03	99.25 : 0.75	1	250	4	standard	292	-	-
04a*	standard	1.3	250	4.6	standard	-	290	330
05	standard	1.25	250	4	Jasco Fine Sil-5 5 µm	-	290	330
06	standard	1.0	250	4	standard	-	290	330
07a**	standard	1.3	250	4.6	Partisil PXS 5/25	-	290	330
08	standard	1.3	250	4	standard	292	-	-
09	99.8 : 0.2	0.7	200	3	CP-Spher Chrompak 8 µm	-	290	330
10	99.8 : 0.2	0.8	250	?	standard	-	290	330
11	standard	1.2	250	3.2	Nucleosil 50 RSL 10 µm	-	290	330
12	standard	1.5	250	4	standard	292	-	-
13	standard	1.0	250	4.6	Spherisorb Si 5 µm	-	290	330
14	standard	1.0	150	4	standard	292	-	-
15	standard	1.5	250	4	standard	-	310	340
16	90 hex:10 THF	1.5	250	4.6	Partisil 10 PAC 10	-	290	330
17a	99.4 : 0.6	1.3	250	4.6	standard	-	290	330
17b	99.4 : 0.6	1.0	120	4.6	standard	-	290	330
18	99 : 1	0.7	300	3.2	Separon SIX 5 µm	292	-	-
19	standard	0.7	250	4.6	Spherisorb S5W	-	295	330

Notes: x"standard" indicates mobile phase hexane/propanol 99.5:0.5 v/v used
*Lab 4 also reported results (4b in Table 2) with Partisil PAC column and 94:6 hexane : THF
**Lab 7 also reported results (7b in Table 2) with Partisil PAC column and 93:7 hexane : THF

TABLE 5. RESULTS OBTAINED USING FLUORESCENCE DETECTION (1985/86) (expressed in µg/g)

	Sample 1				Sample 2				Sample 3			
Tocopherol:	alpha	beta	gamma	delta	alpha	beta	gamma	delta	alpha	beta	gamma	delta
mean value	75	14	504	243	65	215	265	118	135	9	574	127
mean value [all]*	69	17	508	263	69	217	225	130	124	12	603	134
CV_R(%)	18	23	2	5	12	5	9	12	14	24	3	6
CV_R(%) [all]*	25	31	7	17	19	26	24	20	24	19	10	14

***Note:** "all" indicates combined results from both fluorescence and UV detection.

TABLE 6. RESULTS FOR TOCOPHEROLS (µg/g) in MARGARINE SAMPLES A and C (1986/87)

Lab Code	alpha-tocopherol A	alpha-tocopherol m	gamma-tocopherol A	gamma-tocopherol m	delta-tocopherol A	delta-tocopherol m
	C		C		C	
01	89	80	28	24	38	34
	71		19		30	
02	97	98	20	21	30	36
	99		21		36	
03	109	112	23	23	39	39
	114		22		39	
04	103	100	15	16	31	32
	96		17		32	
05	89	108	16	17	34	39
	126		18		44	
06	116	114	22	22	40	40
	112		21		40	
07	41	47	20	21	33	35
	53		22		37	
08	71	87	21	22	25	28
	102		22		30	
09	65	62	16	16	29	28
	59		16		26	
10	94	81	26	22	64	55
	68		18		45	
overall mean		94 (not Lab 7)		20		35

Lab Code	alpha-tocopherol B	alpha-tocopherol m	gamma-tocopherol B	gamma-tocopherol m	delta-tocopherol B	delta-tocopherol m
	D		D		D	
01	11	10	8	14	69	71
	9		20		73	
02	9	10	16	17	72	71
	10		17		69	
03	9	9	17	18	72	73
	9		18		73	
04	10	9	14	15	63	63
	8		15		63	
05	5	7	13	14	77	77
	7		15		77	
06	11	12	19	20	78	85
	12		21		91	
07	8	6	13	14	56	59
	4		14		61	
08	9	10	21	23	63	64
	10		24		65	
09	32	48	13	14	58	60
	63		16		62	
10	11	9	19	16	110	87
	7		12		63	
overall mean		9 (not Lab 9)		16		71

Notes on Table 6:

1) Samples A and C were the same margarine provided as blind duplicates; the alpha-tocopherol content of this margarine was estimated in the co-ordinator's laboratory as being 10 µg/g and a quantity of alpha-tocopherol acetate, equivalent to 85 µg/g alpha-tocopherol, was blended into the margarine so that the expected total level of alpha-tocopherol in samples A and C would be 95 µg/g. The results tabulated in the first column under each tocopherol are the mean results from duplicate injections for each of the blind duplicates. The means tabulated in the second column under each sample are those derived by calculation from the duplicate injection results for each of the blind duplicates.

2) For **alpha-tocopherol** Labs. 1, 7, 8 and 9 technical outliers (level of alpha-tocopherol for one or more results significantly less than the amount of alpha-tocopherol added) and Labs 5 & 10 Cochran outliers; **delta-tocopherol** Lab 10 Cochran outlier; The overall mean value of 94 µg alpha-tocopherol is remarkably close to the expected value of 95 µ/g.

3) All laboratories except 8 and 9 used **fluorescence detection.**

4) Samples B and D were the same margarine provided as blind duplicates; the values tabulated below in the first column under each sample are the mean results from duplicate injections for each of the blind duplicates. The means tabulated in the second column under each sample are those derived by calculation from the duplicate injection results for each of the blind duplicates.

5) For **alpha-tocopherol** Lab 9 Cochran outlier; **gamma-tocopherol** Labs 1 & 10 Cochran outliers; **delta-tocopherol** Labs 6 and 10 Cochran outliers;

Results obtained using fluorescence detection are those indicated in **Table 2** for laboratories 1, 4, 5, 6, 7, 9 and 17. In most instances the mean values for these abstracted results (see **Table 5** below) are in reasonable agreement with those from the complete study, although it will be noted from the data in **Table 5** that the precision is better using fluorescence detection, as indicated by the lower values for the reproducibility standard deviation coefficient of variation.

It has been reported that the fluorescence intensity of each tocotrienol is the same as the corresponding tocopherol [ref 4] and therefore quantitation of these components can be achieved when the corresponding tocopherol standards are available. Most participants separated the major tocotrienols present in **Sample 4**, although not all submitted quantitative results. Alpha-tocotrienol was reasonably well separated from alpha-tocopherol, and the gamma- and delta-tocotrienols were effectively chromatographed.

The laboratories which submitted results obtained when using a Partisil PAC bonded column all achieved good separation of all tocopherols. It will be undoubtedly worth investigating the applications of this column type when it becomes more widely available.

In view of the fact that it was desirable to check if the method could be applied to processed products such as margarines which contained tocopherols in the form of esters it was agreed that a final study of the procedure should be scheduled for 1986/87. It was noted that the precision obtained using the HPLC method studied represents a considerable improvement over that achievable by the present 2.404 method based on TLC/GLC.

4th (FINAL) COLLABORATIVE STUDY AND RESULTS

The purpose of the 1986/87 study, as indicated above, was to examine the applicability of the method (which had proved satisfactory for unprocessed oils and fats) to products such as margarines in which the tocopherols could be present as esters. Two samples of retail-packed margarines, labelled as containing added tocopherols, were selected for the study. To one sample a known amount of alpha-tocopherol was added (see **Note 1** for **Table 6**) in the form of alpha-tocopherol acetate.

Each of the samples were in duplicate and blind coded. Participants were requested to follow the text of the method provided, making two injections of each of the prepared test solutions prepared from the samples. Results submitted by ten laboratories are given in **Table 6**, HPLC working parameters are given in **Table 7**, and a statistical analysis of the results are given in **Tables 8a** and **8b**; values for beta-tocopherol have not been tabulated since this tocopherol was present at a level below 5 μg/g .

Examination of the results indicated that some participants encountered unsatisfactory recoveries of alpha-tocopherol when analysing **Samples A and C**, which had been "spiked" with alpha-tocopheryl acetate. As indicated in **Note 1** for **Table 6** these samples had been spiked with an equivalent of 85 μg/g of alpha-tocopherol and of the twenty results submitted for these samples, seven results (submitted by five laboratories) were significantly lower than this amount.

In view of the fact that thirteen out of twenty results submitted exceeded 85 μg/g alpha-tocopherol (indicating that the tocopheryl acetate added could be successfully recovered in the form of the free tocopherol), the seven results referred to above have been omitted, on technical grounds, for the purposes of the statistical analysis reported for alpha-tocopherol in the second column of figures in Table 8a, although this omission results in insufficient values to arrive at a satisfactory statistical estimate of precision. Of the five laboratories which submitted one or more results significantly below the theoretical level of alpha-tocopherol, three submitted one result with a value above the expected level of 95 μg/g and the mean value of the thirteen results exceeding 85 μg/g is 104 μg/g - a value not far removed from the expected 95 μg/g.

From this it could be concluded that particular care with the saponification step is required to ensure that all tocopherol esters have been converted to the free form. However on the basis of the available data it is not possible to provide valid statistical precision data in the case of the results for the samples containing tocopherol acetate.

It was not considered that a further collaborative study involving specific examination of the saponification procedure could be justified but it was agreed that a short investigation should be carried out by one laboratory. The results of this study were not completely conclusive (see Table 9) although it was evident that special care had to be exercised in the matter of saponification time and temperature in order to ensure that all tocopherol esters were converted to the free form and that no loss of tocopherols occurred. The text of the procedure for the cold saponification step distributed to participants had been reproduced exactly from the protocol adopted for the successful collaborative study of an HPLC method

TABLE 7. HPLC OPERATING PARAMETERS (1986/87)

Lab. No.	Mobile Phase* Hexane/Propanol (99.5:0.5 v/v)	Flow rate ml/min	Column Dimensions length mm	int. dia. mm	Column (standard Lichrosorb SI 60 5 µm)	Detection UV nm	Fl Ex	Fl Em
01	iso-octane/ propanol	2.0	300	4.6	Spherisorb Si 5 µm	-	295	330
02	standard	1.8	250	4.6	standard	-	290	330
03	standard	1.5	250	4.6	Sperisorb Si 5 µm	-	290	330
04	standard	1.5	250	4	Fine SIL-5 5 µm	-	290	330
05	standard	1.0	250	4.6	Zorbax SIL 5 µm	-	290	330
06	standard	1.0	250	4	standard	-	290	330
07	99.4 : 0.6	1.3	250	4.6	standard	-	290	330
08	standard	1	150	3	Separon Six 5 µm	292	-	-
09	99.3 : 0.7	1	250	4	standard	292	-	-
10	standard	1	250	4.6	Spherisorb S5W 5 µm	-	295	330

Notes:

*"standard" indicates hexane/propanol 99.5:0.5 v/v used as mobile phase

All laboratories used 20 µl sample volume except no. 8 (30 µl)

All laboratories used loop injection except nos. 5 & 10

TABLE 8. STATISTICAL ANALYSIS OF RESULTS FOR SAMPLES A AND C AND SAMPLES B AND D

	TABLE 8a. FOR SAMPLES A AND C (1986/87)				TABLE 8b. FOR SAMPLES B AND D (1986/87)		
Tocopherol	alpha	alpha*	gamma	delta	alpha	gamma	delta
Number of laboratories	10	10	10	10	10	10	10
Number of results	20	20	20	20	20	20	20
Number of laboratories retained after elimination of outliers†	8	4*	10	9	9	9	8
Number of accepted results	16	8*	20	18	18	18	16
MEAN VALUE (µg/g)	98	106*	20	34	9	17	67
Repeatability standard deviation (S_r)	14.6	3.4*	1.0	1.9	1.7	1.4	2.2
Repeatability coefficient of variation	42.5%	3.2%*	5.1%	5.6	19.1%	8.2%	3.2
REPEATABILITY VALUE (r) [2.83 x S_r]	41	10	3	5	5	4	6
Reproducibility standard deviation (S_R)	17.1	8.5	2.8	5.3	2.1	3.4	6.8
Reproducibility coefficient of variation	49.5%	8.1%	14.3%	15.6%	23.3%	20.2%	10.2%
REPRODUCIBILITY VALUE (R) [2.83 x S_R]	48	24	8	15	6	10	19

Notes:

Statistical analysis according to ISO 5725-1986
*see comment in text regarding these values
†For details of outliers see notes following Table 6

TABLE 9. SUMMARY OF INVESTIGATION INTO EFFECT OF SAPONIFICATION TIME AND TEMPERATURE

a) Three portions of a sample of margarine containing 16 µg/g (as estimated in the co-ordinator's laboratory) was spiked with 28, 52 and 126 µg/g of alpha-tocopherol (in the form of the acetate ester). The recovery of alpha-tocopherol, following the studied procedure, was 92%, 91% and 91% respectively.

b) A further portion of the same margarine sample, spiked with an equivalent of 52 µg/g of alpha-tocoherol acetate, was saponified at room temperature (25°C), and analysis of the spiked sample gave the following results for different saponification times (min.):

Time	alpha	gamma	delta
5	27	32	66
5	29	34	72
10	53	36	75
10	61	35	72
20	63	36	75
20	63	36	75

c) The same margarine, spiked with alpha-tocopherol acetate as in b), gave the following results when saponified for 10 min. at a higher temperature.:

Temp. °C	alpha	gamma	delta
39	37	34	73
39	32	34	73

The relatively constant values of the results for gamma- and delta-tocopherols confirm that the method is satisfactory for free tocopherols. For alpha-tocopheryl acetate it will be noted that shorter saponification time and higher saponification temperatures than that specified in the method (10 min. and 26°C, respectively) result in low recoveries of alpha-tocopherol from alpha-tocopheryl acetate.

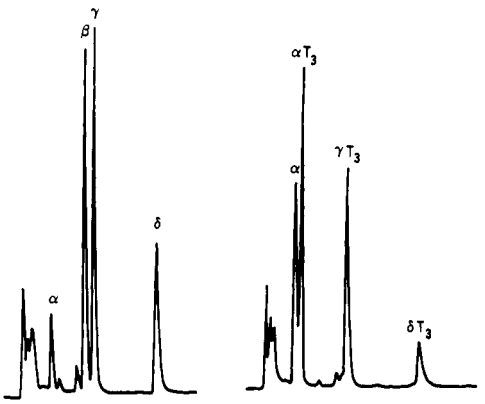

Fig 1. Chromatogram showing the order of elution of tocopherols and tocotrienols

for tocopherols that had been carried out in the Federal Republic of Germany; it was therefore surprising to find participants in the IUPAC study having difficulty in obtaining acceptable recoveries of tocopherols when using this cold saponification procedure.

It was therefore decided to draft the text of the standardised method with the details of the cold saponification procedure being given in the notes to the method (rather than in the main body of the text), together with the caution that low recoveries of tocopherols from tocopherol esters may be experienced if due care is not given to the saponification of the sample. The text of the method would also recommend analysing a particular sample "spiked" with a known amount of tocopherol ester to check the recovery when samples containing tocopherol esters were to be analysed.

Scrutiny of the calculated statistical data indicates that each tocopherol can be determined with similar precision within the range 15 - 600 µg/g and accordingly a statistical table has been drafted for inclusion in the text of the method showing the precision applicable to all tocopherols over this range. The data available for tocotrienols suggests that the method's precision for the determination of these components is similar for that of tocopherols although insufficient data was obtained to provide statistically sound precision estimates.

Following the discussion of the results obtained from the 4th collaborative study and the investigation into the effect of saponification temperature and time on the recovery of alpha-tocopherol from alpha-tocopherol acetate, the Commission decided to adopt the HPLC procedure for the determination of "free" tocopherols, with reference being made in the notes to the method regarding its application to products containing tocopherols esters. The text of the standardised procedure is given on the following pages.

2.432 DETERMINATION OF TOCOPHEROLS AND TOCOTRIENOLS IN VEGETABLE OILS AND FATS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. SCOPE AND FIELD OF APPLICATION

This Standard describes a procedure for the determination of tocopherols and tocotrienols in vegetable oils and fats. The procedure is not directly applicable to processed products such as margarines containing tocopherol esters, but it may be used to determine tocopherols in the unsaponifiable matter obtained from such products (Note 1).

2. PRINCIPLE

Dissolution of the oil or fat (or the unsaponifiable matter obtained from a processed product containing tocopherol esters) in an organic solvent and direct high performance liquid chromatographic (HPLC) separation of the individual tocopherols and tocotrienols. Calibration factors are determined for each tocopherol from the chromatography of solutions of standard tocopherols; calibration factors for tocotrienols are taken to be equivalent to that of the corresponding tocopherols.

3. DEFINITION

The tocopherols and tocotrienols content of an oil or fat is the quantity of tocopherols and tocotrienols, determined in the sample by the described procedure and expressed in micrograms per gram (μg/g).

4. APPARATUS

Note: All glassware should be of low actinic activity.

4.1 HPLC system consisting of a high pressure pump, sample injection device, detector, and chart recorder or recording integrator.
A fluorescence detector should preferably be used with the excitation wavelength set at 290 nm and emission wavelength at 330 nm.
A UV detector may be used if a fluorescence detector is not available. The wavelength of the UV detector should be set at 292 nm.

4.2 HPLC analytical column (250 mm x 4 mm) packed with microparticulate silica having a mean particle size of about 5 μm (Note 2).

4.3 UV spectrometer capable of absolute measurement of absorbance at precisely defined wavelengths.

4.4 Rotary film evaporator

5. REAGENTS

All solvents should be of HPLC grade or equivalent. For additional reagents required when prepared fats containing tocopherol esters are being analysed, see 11.1 - 11.7.

5.1 alpha-, beta-, gamma-, and delta-tocopherol standards (Note 3)

5.2 Methanol

5.3 Dichloromethane

5.4 Hexane

5.5 Propan-2-ol

5.6 HPLC mobile phase, propan-2-ol in hexane (0.5/99.5 v/v)

6. SAMPLING

It is essential that the laboratory sample used for preparing the test sample was taken according to a recognised sampling procedure (such as that described in ISO 5555), and that the laboratory sample was subsequently protected from the effects of heat and light.

7. PROCEDURE

7.1 Preparation of solutions of tocopherol standards

7.1.1 Alpha-tocopherol standard stock solution

Prepare a stock solution of alpha-tocopherol by accurately weighing about 10 mg of the standard (5.1) into a 100 ml volumetric flask and making up to volume with hexane (5.4).

Pipette 10 ml of this solution into an amber glass round bottomed flask and remove all hexane on a rotary evaporator (4.4) at a temperature not higher than 40°C. Restore atmospheric pressure with nitrogen and remove the flask from the evaporator as soon as all the solvent has been removed. Pipette into the flask 10 ml of methanol (5.2) and swirl to dissolve the tocopherol. Measure the absorbance of this solution at 292 nm and calculate the concentration (as µg/ml alpha-tocopherol) by dividing the absorbance value by 0.0076 (Note 4).

7.1.2 Beta-, gamma-, and delta-tocopherol standards stock solutions

Prepare similarly stock solutions and aliquots for UV spectrometry of beta-, gamma and delta-tocopherol standards (5.1) as described in 7.1.1 for alpha-tocopherol. Measure the absorbance of each of these solutions at the following wavelengths and use the corresponding divisor factors (Note 4) for calculation of concentration :

296 nm	beta-tocopherol	= 0.0089
298 nm	gamma-tocopherol	= 0.0091
298 nm	delta-tocopherol	= 0.0087

7.1.3 Mixed tocopherol standards working solution

Mix appropriate volumes of the stock solutions of the tocopherol standards to obtain a mixed tocopherol standards working solution, and dilute with hexane to give a solution containing between 1 and 5 µg per ml of each tocopherol. [Note: a more concentrated solution may have to be prepared if a UV detector is used. It is important that all standards are protected from light and stored refrigerated - see Note (5)].

7.2 Optimisation of working parameters

Condition the column (4.2), if necessary (Note 6).

Pump the propan-2-ol/hexane mobile phase (5.6) through the column at a flow rate of 1 ml/min for at least 30 mins.

Inject about 20 µl of the mixed tocopherol standards working solution (7.1.3) onto the column and if necessary adjust the propan-2-ol content of the mobile phase and the flow rate (Note 7) to achieve the following conditions:

1. alpha-tocopherol retention time not less than 5 mins.
2. resolution factor (R) for the separation of beta- and gamma-tocopherol not less than 1.0, i.e., almost baseline separation (Note 8).

Select the optimum settings for detector and integrator sensitivity and chart speed. Inject about 20 µl of the mixed tocopherol standards working solution (7.1.3). Repeat the injection and check that reproducible chromatograms are obtained.

7.3 Preparation of the test sample

The test sample should be prepared, in the case of liquid laboratory samples, by homogenisation as described in 2.001 except that filtration should be avoided. In the case of solid samples, transfer a representative portion (i.e. not less than 10% by weight of the laboratory sample) to a glass beaker and carefully homogenise by melting, with gentle mixing, in a water bath at a temperature not exceeding 40°C. The preparation of the test sample should be carried out, as far as is practicable, in subdued light and in any case out of direct sunlight.

7.4 Preparation of the test solution

Weigh accurately about 2 g of the prepared test sample (Note 9) into a 25 ml volumetric flask. Add a quantity of hexane (5.4), swirling to dissolve the sample and make up to volume

with the same solvent. If a fluorescent detector is used it may be necessary to make a further dilution of this solution prior to chromatography.

It is important that the test solutions are protected from light prior to analysis, and analysed on the day of preparation.

7.5 HPLC Determination of tocopherols in the test solution

Inject 20 µl of the mixed tocopherol standards working solution (7.1.3) on to the column and record the areas of the tocopherol peaks. If an integrator is not available record peak heights (measured in mm).

Inject 20 µl of the test solution (7.4) on to the column and identify the tocopherols (and tocotrienols) present by reference to the chromatograms obtained from standards (Note 10). Record the areas of the tocopherol peaks (or peak heights). Record the areas of any tocotrienol peaks if these are present and are to be quantified. Duplicate injections should be made.

Inject a further 20 µl of the mixed tocopherol standards working solution (7.1.3) and record the areas of the tocopherol peaks.

7.6 Number of determinations

Carry out two determinations (each consisting of duplicate injections of the prepared test solutions) in rapid succession, using a fresh test portion for each determination.

8. CALCULATION AND EXPRESSION OF RESULTS

8.1 The alpha-tocopherol content of the sample is given by:

$$\frac{C \times a \times D \times 25}{A \times m} \quad \mu g/g$$

where C = concentration of the alpha-tocopherol standard (µg/ml)

A = mean of the peak areas obtained for the alpha-tocopherol standard

a = mean of the peak areas obtained for the alpha-tocopherol in test sample

m = mass of test sample taken

D = dilution factor*

*e.g., for a test solution prepared from a 1:10 dilution of a 25 ml solution of the test portion this factor would be 10.

8.2 The beta-, gamma-, and delta-tocopherol contents of the test sample are calculated in the same way using the data obtained from chromatography of the corresponding tocopherol standard (Note 11).

8.3 The tocotrienol content of a sample can be estimated using the C and A values for the corresponding tocopherol (Note 12).

8.4 Report as the final result the mean of the values obtained from the two determinations, provided the requirements for repeatability (8.1) are met. If the requirements for repeatability are not met, carry out a further two determinations on the test sample. If the range ($x_{max} - x_{min}$) of the four values obtained is < 1.3 x r*, report as the final result the mean of the four values; otherwise report as the final result the median of the four values, i.e. the mean of the two intermediate values. *[where r = the repeatability value - see - the TABLE in 9.3 below.]

8.5 Report the results for each tocopherol and tocotrienol to the nearest µg.

9. PRECISION

The results of an interlaboratory study organised at the international level gave the statistical results which are summarised in the TABLE in 9.3 below.

9.1 Repeatability

When the mean of the values obtained from two single determinations carried out in rapid succession by the same operator, using the same apparatus under the same conditions for the analysis of the same test sample, lies within the range of the mean values cited in the **TABLE** in 9.3, the difference between the two values obtained should not be greater than the **repeatability value (r)**, which can generally be deduced by linear interpolation from the **TABLE** in 9.3.

9.2 Reproducibility

When the values for the final result, obtained by operators in different laboratories using different apparatus under different conditions, from the analysis of the same laboratory sample, lie within the range of mean values cited in the **TABLE** in 9.3, the difference between the values for the final result obtained by those operators should not be greater than the **reproducibility value (R)** [Note 13], which can generally be deduced by linear interpolation from the **TABLE** in 9.3.

9.3 TABLE - STATISTICAL ANALYSIS OF RESULTS FOR TOCOPHEROLS (expressed as µg/g)

Level	A	B	C	D
Number of laboratories	16	17	16	17
Number of results	32	34	32	34
Number of laboratories retained after elimination of outliers*	14	15	13	16
Number of accepted results	28	30	26	32
MEAN VALUE (µg/g)	17	69	263	508
Repeatability standard deviation (S_r)	0.8	3.5	5.3	12.6
Repeatability coefficient of variation	5%	5%	2%	2.5%
REPEATABILITY VALUE (r) [2.83 x S_r]	2	10	15	36
Reproducibility standard deviation (S_R)	5.1	17.5	44.5	36.0
Reproducibility coefficient of variation	31%	25%	17%	7%
REPRODUCIBILITY VALUE (R) [2.83 x S_R]	15	49	126	102

Statistical analysis according to ISO 5725-1986

Values for reproducibility value (R) are those applicable to results obtained from single determinations (Note 13)

10. NOTES

1. It is recommended that the unsaponifiable matter is obtained by a method involving a cold saponification procedure such as that described in the **APPENDIX**. Particular attention must be paid to saponification temperature and time, otherwise low recoveries of tocopherols from tocopherol esters may be obtained.

2. Suitable silica column packing materials are 5 µm LiChrosorb SI 60 or Spherisorb S5W.

3. Beta-, gamma-, and delta-tocopherol standards can be obtained from Merck; alpha-tocopherol can be obtained from various suppliers. It has been reported that the purity of some commercially available tocopherol standards may vary between 85 and 100 per cent (Ref. 1). This confirms the importance of determining the concentration of prepared standard solutions by UV spectrometry.

4. The divisor factors quoted for the tocopherols are derived from their E values (1%/1 cm) quoted in Reference 2. For example the E value (1%/1 cm) of alpha-tocopherol is 76 at 292 nm (in methanol); therefore a 1 µg/ml solution of alpha-tocopherol will have an absorbance of 0.0076 at 292 nm.

5. Stock standard solutions can be satisfactorily stored in amber low actinic glassware for up to a week if refrigerated. Working standard solutions must be prepared each working day. Protection from light is of the utmost importance.

6. If the column (4.2) is new or of unknown history, wash and condition for about ten minutes with methanol, then dichloromethane, followed by hexane at a flow rate of about 1 ml/min.

7. Mobile phase flow rates in the range 0.7 to 1.5 ml/min have been found to be satisfactory. Higher flow rates can result in poor chromatography which must be avoided when UV detection is used.

8. The resolution factor (R) is calculated from :

$$R = \frac{R_{d1} - R_{d2}}{0.5\,[W_1 + W_2]}$$

where R_{d1} = retention distance of gamma-tocopherol

R_{d2} = retention distance of beta-tocopherol

W1 = width at base of gamma-tocopherol peak

W2 = width at base of beta-tocopherol peak

It should be possible to achieve an efficiency of 10,000 plates per metre calculated on the delta-tocopherol peak. The efficiency (n), in plates per metre, may be calculated from:

$$n = 5.54\,[R_{d3}/w_h]^2$$

where R_{d3} = retention distance of delta-tocopherol

w_h = peak width at half height

9. When analysing processed products such as margarines, and samples containing added tocopherol esters, a cold saponification procedure must be performed prior to chromatography. [Note: When samples contain tocopherol esters, parallel samples spiked with known amounts of alpha-tocopherol acetate should be analysed to enable a check to be made on the recovery of tocopherols from tocopherol esters.] The saponification procedure is described in the **APPENDIX**.

10. If any tocopherol standards are not available, a blend of wheat germ and soya bean oil can be used to obtain chromatograms which contain alpha-, beta-, gamma-, and delta-tocopherols. These can be used to assist peak identification in test sample chromatograms.

Palm oil can be used to identify alpha- and gamma-tocotrienols if required. The following relative retention times have been found to be typical :

alpha-tocopherol = 1.0	beta-tocopherol = 1.6
delta-tocopherol = 1.7	gamma-tocopherol = 3.0

11. If fluorescence detection is used and the only standard available is alpha-tocopherol, relate all tocopherols to the alpha-tocopherol standard, but make this clear when reporting results. If UV detection is used, again relate all tocopherols to the alpha-tocopherol standard, but normalise the peak areas to alpha-tocopherol using the divisor values given in 7.1.1 and 7.1.2.

12. According to the literature the fluorescence intensity of tocotrienols is the same as the corresponding tocopherol [Ref. 5], and the UV absorbancies are similar [Ref. 2].

13. It should be noted that the reproducibility values (R) cited in the **TABLE** in 9.3 apply in the particular case when the results of single determinations obtained by two laboratories are being compared. When following the method described and it is desired to compare the final results (which have been derived from the means of duplicate determinations) obtained by two laboratories, the values for (R) should be converted to the 95% probability critical difference values (CrD_{95}) applicable to the means of two determinations, using the formula:

$$CrD_{95} = \sqrt{[R^2 - r^2/2]}$$

APPENDIX

Cold saponification procedure for samples containing added tocopherol esters

A1. Reagents:

A1.1 Ethanol, approx. 96 % pure
A1.2 Ethanol, absolute, 99% pure
A1.3 Pyrogallol
A1.4 Potassium hydroxide solution, aqueous, 60% (m/m)
A1.5 Diethyl ether, peroxide free containing 0.1% (m/m) pyrogallol
A1.6 Hydrochloric acid, 0.01 mol per litre
A1.7 Sodium sulphate, anhydrous

A2. Procedure

Weigh accurately about 2 g of the prepared sample (6.2) in to a 100 ml flat-bottomed flask and thoroughly disperse the molten test portion in approximately 8 ml of ethanol (A1.1) by gentle swirling. Add 100 mg of pyrogallol (A1.3) and swirl to dissolve. Purge the flask with nitrogen, add 4 ml of potassium hydroxide solution (A1.4), re-purge the flask with nitrogen, and close with a glass stopper. Place the flask in a 26°C water bath and shake vigorously for ten minutes. All operations must be performed in the absence of direct sunlight - use amber glassware or shield with aluminium foil.

Add 50 ml of deionised water to the flask and transfer contents quantitatively to a 250 ml separating funnel. Wash the flask with 50 ml of diethyl ether (A1.5) and transfer the washings to the funnel. Shake the separator vigorously for one minute, releasing the pressure occasionally. Allow the layers to separate and draw off the lower aqueous layer. Extract the aqueous layer a further four times with 30 ml aliquots of diethyl ether and combine the ether extracts.

Wash the combined diethyl ether extracts with 50 ml of water (shaking carefully to avoid emulsion formation) and then with 30 ml of dilute hydrochloric acid (A1.6). Add about 3 g of anhydrous sodium sulphate (A1.7) with gentle mixing to absorb water. Filter the ether extracts through a phase-separating paper and collect the filtrate in a round-bottomed amber rotary evaporator flask. Remove the ether under reduced pressure (4.4) at a temperature of not more than 40°C. If a liquid residue remains in the flask add ethanol (A1.2) and re-evaporate to dryness. Wash the sides of the flask with hexane (5.4) and transfer the contents quantitatively to a 50 ml volumetric flask and bulk to volume. Make a suitable dilution of the prepared test solution (as described in 6.3) and proceed to 6.4.

* * * *

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